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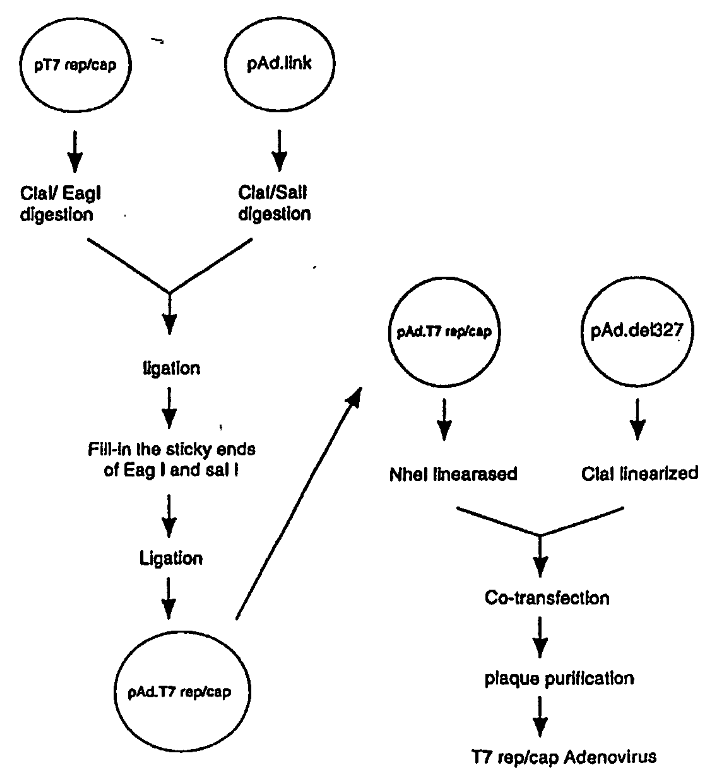
(54) Title: AN INDUCIBLE METHOD FOR PRODUCTION OF RECOMBINANT ADENO-ASSOCIATED VIRUSES UTILIZING T7 POLYMERASE

(57) Abstract

Methods for efficient production of recombinant AAV are described. In one aspect, three vectors are introduced into a host cell. A first vector directs expression of T7 polymerase. A second vector carries rep and cap under the control of the T7 promoter. A third vector contains a rAAV cassette which contains a minigene flanked by AAV ITRs. In a second aspect, the host cell is stably transfected to contain a plasmid bearing one of the required vector components and the host cell is double transfected/infected.



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AN INDUCIBLE METHOD FOR PRODUCTION OF RECOMBINANT ADENO-ASSOCIATED VIRUSES UTILIZING T7 POLYMERASE

Background of the Invention

Adeno-associated virus is a replication-deficient parvovirus, the genome of which is about 4.6 kb in length, including 145 nucleotide inverted terminal repeats (ITRs). The single-stranded DNA genome of AAV contains genes responsible for replication (rep) and formation of virions (cap).

When this nonpathogenic human virus infects a human cell, the viral genome integrates into chromosome 19 resulting in latent infection of the cell. Production of infectious virus and replication of the virus does not occur unless the cell is coinfected with a lytic helper virus such as adenovirus or herpesvirus. Upon infection with a helper virus, the AAV provirus is rescued and amplified, and both AAV and helper virus are produced.

AAV possesses unique features that make it attractive as a vector for delivering foreign DNA to cells. Various groups have studied the potential use of AAV in the treatment of disease states.

However, an obstacle to the use of AAV for delivery of DNA is the lack of highly efficient methods for encapsidation of recombinant genomes. See, R. Kotin, Hum. Gene Ther., 5:793-801 (1994). Furthermore, the rep gene product is toxic to cells and thus cannot be expressed at high levels. For example, previously known methods employ transfection of host cells with a rAAV genome which lacks rep and cap genes followed by co-infection with wild-type AAV and adenovirus. However, this method leads to unacceptably high levels of wild-type AAV. Incubation of cells with rAAV in the absence of contaminating wild-type AAV or helper adenovirus is associated with little recombinant gene expression. And, in the absence of the AAV rep gene product, integration is inefficient and not directed to chromosome 19.

Bacteriophage T7 RNA polymerase (T7 Pol) is the product of T7 gene 1, which can recognize its responsive promoter sequence specifically and exhibit a high transcriptase activity [M. Chamberlin et al, Nature, 228:227-231 (1970); J. Dunn and F. Studier, J. Mol. Biol., 166:447-535 (1983); and B. Moffatt et al, Cell, 49:221-227 (1987)]. It has been used for heterologous expression of proteins in *E. coli* [S. Tabor and C. Richardson, Proc. Natl. Acad. Sci. USA, 82:1074-1078 (1985); F. Studier and B. Moffatt, J. Mol. Biol., 189:113-130 (1986)], in recombinant vaccinia virus-infected eukaryotic cells [T. Fuerst et al, Proc. Natl. Acad. Sci. USA, 83:8122-8126 (1986); A. Ramsey-Ewing and B. Moss, J. Biol. Chem., 271:16962-16966 (1996)], and in mammalian cells [A. Lieber et al, Nucl. Acids Res., 17:8485-8493 (1989)].

What is needed is an efficient method for production of rAAV which avoids the problems associated with rep toxicity for the packaging cell.

Summary of the Invention

The present invention provides an inducible method for efficient production of rAAV which makes use of T7 polymerase. T7 Pol is derived from lambda phage and its promoter is not active in mammalian cells. Thus, expression of rep/cap can be controlled by placing these genes under control of the T7 promoter and providing the T7 Pol in trans or under the control of an inducible promoter. Thus, this method avoids the toxic effects of rep which rendered prior art methods of producing rAAV inefficient. The method of the invention is particularly suitable for large scale production of rAAV, which is desired for rAAV vectors to be used in gene therapy.

In one aspect, the invention provides a method of producing rAAV which utilizes three vectors. A first

vector is capable of expressing T7 polymerase in the host cell following transfection or infection. A second vector comprises the AAV *rep* and *cap* genes under the control of T7 promoter sequences (T7/rep/cap). The third vector comprises a cassette containing 5' and 3' AAV inverted terminal repeats (ITRs) flanking a selected transgene. A host cell containing these three vectors is cultured under conditions which permit replication and packaging of a recombinant AAV, and the rAAV is recovered.

In another aspect, the invention provides a method in which a host cell is stably transfected with one of the three components of the system used in the triple infection system. The remaining components are introduced into the host cell, as described above.

In one embodiment, the invention provides method in which a vector containing T7/*rep*/*cap* and a vector containing a cassette comprising a selected minigene flanked by 5' and 3' AAV ITRs are introduced into a host cell expressing T7 polymerase. The host cell is then cultured under conditions which permit production of rAAV. In another embodiment, this invention provides a method which utilizes a host cell stably transfected with a plasmid containing T7/*rep*/*cap*. A vector containing T7 pol and a vector containing a cassette comprising 5' AAV inverse terminal repeat (ITR), a selected minigene, and 3' AAV ITR are introduced into the host cell. The host cell is cultured under conditions which permit production of rAAV. In still another embodiment, the invention provides a method which utilizes a host cell stably transfected with a rescuable rAAV cassette. A vector containing T7 pol and a vector containing T7/*rep*/*cap* are introduced into the host cell. The host cell is cultured under conditions which p rmit production of rAAV.

In yet another aspect, the present invention provides a method which utilizes a host cell stably transfected with two of the three components of the system used in the triple infection system. The remaining component is then introduced into the host cell, as described above.

In a further aspect, the present invention provides a method which utilizes a host cell stably transfected with the three components of the system used in the triple infection system. In this aspect, the T7 Pol is controlled by an inducible promoter.

In still a further aspect, the invention provides a rAAV produced according to the method of the invention.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

Fig. 1 provides a schematic illustration of the construction of a recombinant adenovirus containing the T7 polymerase gene.

Fig. 2 provides a schematic illustration of the construction of a recombinant plasmid containing the AAV rep/cap genes under control of a T7 promoter.

Fig. 3 provides a schematic illustration of the construction of a recombinant adenovirus containing the rep/cap genes under control of a T7 promoter.

Fig. 4 provides a schematic illustration of the construction of a recombinant hybrid Ad/AAV virus.

Detailed Description of the Invention

The invention provides an inducible method for efficient production of recombinant AAV vectors useful particularly for gene delivery and transfer.

Specifically, the invention provides methods of AAV production in which expression of the toxic but necessary rep gene is controlled by the T7 promoter.

Thus, in one aspect, the method of the invention for production of rAAV involves introducing into a host cell the AAV rep and cap genes under control of a T7 promoter, and a recombinant adeno-associated virus (rAAV) cassette containing a selected minigene flanked by AAV ITRs. Upon introduction of a gene encoding T7 pol, high level expression of rep protein from the T7/rep/cap construct is induced and cells may be grown on a large scale. When rep expression is desired, the cells are caused to express the T7 polymerase which acts on the T7 promoter. This facilitates the efficient replication and packaging of rAAV carrying a gene of interest.

A host cell may be triple transfected (or infected) with vectors containing the above elements. Alternatively, a host cell which expresses one or more of the required elements and may be transfected/infected with the remaining elements is utilized. In another alternative, a host cell is utilized which stably expresses all three elements of the system, and the T7 pol is placed under the control of an inducible promoter, which permits rep/cap expression to be controlled and the avoidance of toxic effects to the cell.

For each of the vector components used in the method of the invention, adenoviral constructs are currently preferred. However, using the information provided herein and known techniques, one of skill in the art could readily construct a different viral (adenoviral or non-adenoviral) or a plasmid vector which is capable of driving expression of the desired genes in the host cell. For example, although less preferred because of their inability to infect non-dividing cells, vectors

carrying the required elements of this system, e.g., the T7 polymerase, may be readily constructed using retroviruses. Therefore, this invention is not limited by the virus or plasmid selected for purposes of introducing the T7 pol, T7/rep/cap, or AAV cassette into the host cell. Desirably, at least one of the vectors is a virus which provides the necessary helper functions to enable packaging. Alternatively, the helper functions may be provided by a co-transfected adenovirus or herpesvirus. Suitable techniques for introducing these vectors into the host cell are discussed below and are known to those of skill in the art. As used herein, a "host cell" is any cell (cell line), preferably mammalian, which permits expression of the T7 pol and T7/rep/cap and packaging of the rAAV containing the cassette, under the conditions described herein. Suitable packaging cells are known, and may be readily selected by the skilled artisan.

A. *Triple Infection/Transfection*

As stated above, a host cell used for assembly and packaging of recombinant AAV may be transfected with plasmid vectors or infected with viral vectors containing the required components of the system.

1. *T7 Pol Vectors*

In a preferred embodiment, a first vector contains the T7 Pol gene under the control of a suitable promoter. In example 5 below, the nuclear localized T7 Pol gene is obtained from a publicly available plasmid [M. Strauss, <u>Nucleic Acid Res.</u>, <u>17</u>:8485-8493 (1989)]. However, the gene may alternatively be obtained from other commercial and academic sources, including the American Type Culture Collection (pTF7-3, Accession No. 484944). See, also GenBank accession number M30308. Desirably, the T7 pol

gene is linked to a nuclear localization signal, such as that described in Dunn, Gene, 68:259-266 (1988), using conventional techniques.

Desirably, T7 Pol is under the control of a cytomegalovirus (CMV) immediate early promoter/enhancer [see, e.g., Boshart et al, Cell, 41:521-530 (1985)]. However, other suitable promoters may be readily selected by one of skill in the art. Useful promoters may be constitutive promoters or regulated (inducible) promoters, which will enable control of the amount of the transgene to be expressed. For example, another suitable promoter includes, without limitation, the Rous sarcoma virus LTR promoter/enhancer. Still other promoter/enhancer sequences may be selected by one of skill in the art.

In addition, the vector also includes other conventional regulatory elements necessary to drive expression of T7 Pol in a cell transfected with the vector. Such regulatory elements are known to those of skill in the art.

2. *T7/Rep/Cap Vectors*

The second vector component of this system contains the *rep* and *cap* genes under control of a T7 promoter. The *rep* and *cap* genes can be obtained from a variety of known sources. See, e.g., T. Shenk, J. Virol., 61:3096-3101 (1987), which provides the AAV2 genome within the plasmid p*sub*201; E. W. Lusby et al, J. Virol., 41:518-526 (1982) and J. Smuda and B.J. Carter, Virology, 184:310-318 (1991).

Similarly, the T7 promoter sequences [J. J. Dunn and F.W. Studier, J. Mol. Biol., 166:477-535 (1983) may be obtained from a variety of commercial and academic sources. In a preferred embodiment, the vector further contains the sequence of untranslated region (UTR) of encephalomyocarditis (EMCV) downstream of the T7

promoter. The inventors believe this sequence increases expression of the gene 5- to 10-fold.

In addition, the vector also includes conventional regulatory elements necessary to drive expression of the rep/cap in a cell transfected with the vector. Such regulatory elements are known to those of skill in the art.

3. *rAAV Cassette (Template)*

The third vector component contains a rAAV cassette containing a minigene flanked by AAV ITRs. As discussed in more detail below, such a minigene contains a suitable transgene, a promoter, and other regulatory elements necessary for expression of the transgene.

The AAV sequences employed are preferably limited to the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences [See, e.g., B. J. Carter, in "Handbook of Parvoviruses", ed., P. Tijsser, CRC Press, pp.155-168 (1990)]. Desirably, substantially the entire 143 bp sequences encoding the ITRs are used in the vectors. Some degree of minor modification of these sequences is expected to be permissible for this use. The ability to modify these ITR sequences is within the skill of the art. See, e.g., texts such as Sambrook et al, "Molecular Cloning. A Laboratory Manual.", 2d edit., Cold Spring Harbor Laboratory, New York (1989). Alternatively, it may be desirable to use functional fragments of the ITRs. Such fragments may be determined by one of skill in the art.

The AAV ITR sequences may be obtained from any known AAV, including presently identified human AAV types. Similarly, AAVs known to infect other animals may also be employed in the vector constructs of this invention. The selection of the AAV is not anticipated to limit the following invention. A variety of AAV

strains, types 1-4, are available from the American Type Culture Collection or available by request from a variety of commercial and institutional sources. In the following exemplary embodiment an AAV-2 is used for convenience.

The 5' and 3' AAV ITR sequences flank a minigene which is made up of a selected transgene sequence and associated regulatory elements. The transgene sequence of the vector is a nucleic acid sequence heterologous to the AAV sequence, which encodes a polypeptide or protein of interest. The transgene is operatively linked to regulatory components in a manner which permits transgene transcription.

The composition of the transgene sequence will depend upon the use to which the resulting vector will be put. For example, one type of transgene sequence includes a reporter sequence, which upon expression produces a detectable signal. Such reporter sequences include without limitation an *E. coli* beta-galactosidase (*LacZ*) cDNA, an alkaline phosphatase gene and a green fluorescent protein gene. These sequences, when associated with regulatory elements which drive their expression, provide signals detectable by conventional means, e.g., ultraviolet wavelength absorbance, visible color change, etc. A more preferred transgene sequence includes a therapeutic gene which expresses a desired gene product in a host cell. These therapeutic nucleic acid sequences typically encode products which may be administered to a patient *in vivo* or *ex vivo* to replace or correct an inherited or non-inherited genetic defect or treat an epigenetic disorder or disease. The selection of the transgene sequence is not a limitation of this invention.

In addition to the major elements identified above, the minigene also includes conventional regulatory elements necessary to drive expression of the transgene in a cell transfected with the vector carrying the AAV cassette. Thus the minigene contains a selected promoter which is linked to the transgene and located within the minigene, between the AAV ITR sequences of the vector.

Selection of the promoter which mediates expression of the transgene is a routine matter and is not a limitation of the vector. Useful promoters include those which are discussed above in connection with the first vector component.

The minigene will also desirably contain heterologous nucleic acid sequences including sequences providing signals required for efficient polyadenylation of the transcript and introns with functional splice donor and acceptor sites. A common poly-A sequence which is employed in the exemplary vectors of this invention is that derived from the papovavirus SV-40. The poly-A sequence generally is inserted following the transgene sequences and before the 3' AAV ITR sequence. A common intron sequence is also derived from SV-40, and is referred to as the SV-40 T intron sequence. A minigene of the present invention may also contain such an intron, desirably located between the promoter/enhancer sequence and the transgene. Selection of these and other common vector elements are conventional and many such sequences are available [see, e.g., Sambrook et al, and references cited therein].

The rAAV vector containing the AAV ITRs flanking the minigene may be carried on a plasmid backbone and used to transfect a selected host cell or may be flanked by viral sequences (e.g., adenoviral sequences) which permit it to infect the selected host

cell. Suitable Ad/AAV recombinant viruses may be produced in accordance with known techniques. See, e.g., WO 96/13598, WO 95/23867, and WO 95/06743, which are incorporated by reference herein.

B. *Double Infection/Transfection*

A cell line which stably expresses T7 pol may be constructed, and then double transfected (or infected) with a vector containing T7/rep/cap and a vector containing a rAAV cassette, as illustrated in the following table (Inf = infection and Txf = transfection).

	T7 rep/cap	rAAV
System A	Inf	Inf
System B	Inf	Txf
System C	Txf	Inf
System D	Txf	Txf

Alternatively, a cell line stably transfected with T7 rep/cap may be double transfected (infected) with a vector carrying T7 pol and a vector carrying the rAAV cassette, as illustrated in the following table.

	T7 Pol	rAAV
System E	Inf	Inf
System F	Inf	Txf
System G	Txf	Inf
System H	Txf	Txf

In still another alternative, a cell line which contains a rescuable rAAV cassette may be double transfected (infected) with a vector containing T7 Pol and a vector containing T7/rep/cap, as illustrated in the following table.

	T7 Pol	T7 rep/cap
System I	Inf	Inf
System J	Inf	Txf
System K	Txf	Inf
System L	Txf	Txf

The plasmid and viral vectors used in double transfection/infection steps are as described above in connection with the triple transfection and/or infection system.

A stable cell line of the invention can be produced by transfection of a desired cell, e.g., 293 cells or other packaging cell lines expressing required adenoviral genes, with a plasmid containing the desired gene, e.g., T7 Pol, using conventional techniques and selected via an accompanying resistant marker gene. Depending upon whether inducible or constitutive expression is desired, an appropriate promoter may be selected. For example, if a host cell inducibly expressing T7 Pol is desired, the cell may be transfected with a plasmid containing T7 Pol under control of a metallothionein promoter. Alternatively, if a host cell constitutively expressing T7 Pol is desired, it may be inserted under control of a RSV or CMV promoter. Similar techniques may be used for providing a host cell containing the T7/rep/cap and a host cell containing a rescuable rAAV. The examples below describe production of stable cell lines. However, one of skill in the art could readily produce such cell lines using other conventional techniques. See, generally, Ausubel et al, Current Protocols in Molecular Biology (Wiley Interscience 1987).

C. *Single Infection/Transfection*

A cell line which stably expresses two of the components of this system may be constructed, and then transfected (or infected) with a vector containing the remaining component of the system, as described above. For example, using the techniques described herein, a cell line is utilized which is stably transfected with the T7/rep/cap and a rescuable rAAV. The cell line is then transfected or infected with a

vector containing the T7 pol. As another example, the cell line is stably transfected with the T7 pol and a rescuable rAAV. The cell line is then transfected or infected with a vector containing the T7 rep/cap.

D. *Cell Line Containing T7 Pol, rAAV and T7/rep/cap*

A cell line which stably expresses all three of the components of this system may be constructed and utilized in the method of the invention. Using known techniques, a suitable packaging cell line is constructed which contains the rAAV, the T7/rep/cap and the T7 pol. In this embodiment, the T7 Pol is placed under the control of an inducible promoter. Suitable inducible promoters are known to those of skill in the art and are discussed herein. For example, T7 Pol may be placed under control of a metallothionein promoter. In this manner, expression of the T7 Pol, and thus the rep/cap, which are under control of the T7 promoter can be regulated and toxic effects to the cell avoided.

E. *Production of Vectors and rAAV*

Assembly of the selected DNA sequences of the adenovirus, AAV and the reporter genes or therapeutic genes and other vector elements into the vectors described above utilize conventional techniques. Such techniques include cDNA cloning such as those described in texts [Sambrook et al, cited above], use of overlapping oligonucleotide sequences of the adenovirus or AAV genome, polymerase chain reaction, and any suitable method which provides the desired nucleotide sequence.

Whether using the three vector system, or stably infected cells, introduction of the vectors into the host cell is accomplished using known techniques. Where appropriate, standard transfection and co-transfection techniques are employed, e.g., $CaPO_4$

transfection techniques using the complementation human embryonic kidney (HEK) 293 cell line (a human kidney cell line containing a functional adenovirus E1a gene which provides a transacting E1a protein). Other conventional methods employed in this invention include homologous recombination of the viral genomes, plaquing of viruses in agar overlay, methods of measuring signal generation, and the like.

Following infection/transfection, the host cell is then cultured under standard conditions, to enable production of the rAAV. See, e.g., F. L. Graham and L. Prevec, Methods Mol. Biol., 7:109-128 (1991). Desirably, once the rAAV is identified using conventional techniques, it may be isolated using standard techniques and purified.

These examples illustrate the preferred methods of the invention. These examples are illustrative only and do not limit the scope of the invention.

Example 1 - Construction of a T7 Pol Adenovirus

Figure 1 provides a schematic of the construction of the recombinant adenovirus carrying the T7 polymerase.

The plasmid pMTT7N was obtained from Dr. Michael Strauss [A. Lieber et al, Nucl. Acids Res., 17:8485-8493 (1989)]. pMTT7N contains a N-terminal nuclear location signal of SV40 large T antigen fused to the T7 Pol gene (T7N Pol) which is linked to the polyadenylation sequence of SV40. Expression is driven by the inducible mouse metallothionein promoter.

The pMTT7N plasmid DNA was digested with BglII and PvuII restriction enzymes and the fragments separated on an agarose gel. The BglII/PvuII T7 Pol DNA fragment was ligated to the BglII/EcoRV cleaved vector pAd.CMV.link.1 to form pAd.CMV.T7N. pAd.CMV.link.1 is a

plasmid containing the adenoviral sequences 0 to 16 map units deleted of E1a and E1b into which a CMV promoter-polylinker cassette was cloned. This is described in X. Ye et al, J. Biol. Chem., 271:3639-3646 (1996).

In pAd.CMV.T7N, the expression unit of T7 Pol is directed by the CMV promoter. The promoter for the T7 Pol gene is linked to a PolyA tail as a cassette within the sequence of adenovirus 0-1 map unit (mu) and 9-16 mu. The pAd.CMV.T7N is linearized by Nhe I digestion and co-transfected with Cla I linearized Addel327 backbone using Cellphate kit (Pharmacia). Approximately 1 week post-transfection, the T7 Pol adenovirus can be isolated from the plaques for further purification.

Example 2 - Cell Lines Expressing T7 Pol

A cell line stably expressing T7 Pol is established by co-transfection of plasmids pMTT7N and pMTCB6+ (which provides a selective marker) [K. H. Choo et al, DNA, 5:529-538; Eur. J. Biochem., 174:417-424] into 293 cell at a ratio of 10:1 using calcium phosphate precipitation [F. Graham and A. van der Eb, Virol., 52:456-467 (1973)]. Colony cloning is carried out by Geneticin selection at a concentration of 1 mg/ml. Each clone obtained is transfected with pT7 rep/cap plasmid [see, Example 3 below] and analyzed for its ability to induce the expression of Rep protein upon induction by supplementation with Zn^{++}.

To establish a stable cell line that constitutively expresses the T7 Pol, the T7N Pol (obtained by BglII/PvuII digestion of pMTT7N, as described above) was subcloned downstream of RSV promoter at the cloning sites of BamHI and PvuII in the vector of pEBVhis [Invitrogen]. The resulting plasmid, designated pEBVhisT7N, was transfected into 293 cells and selected

with Hygromycin at a concentration of 400 μg/ml. Each positive clone is analyzed for the presence of T7 Pol by its ability to produce expression of T7-LacZ or T7-rep/cap in cells transfected with these plasmids.

Example 3 - Production of T7 rep/cap Adenovirus

The production of this recombinant adenoviral vector is illustrated schematically in Figs. 2 and 3.

A. *Plasmid Construction*

The plasmid pTM1 [B. Moss et al, Nature, 348:91-92 (1990)], designed for expressing genes under control of the T7 promoter/EMCV UTR (untranslated region of encephalomyocarditis), was used as the vector for expressing AAV rep/cap. The entire coding sequence of rep/cap was separated into two portions by the unique SacI site and subcloned into the pTM1 plasmid as described below.

Because there is no appropriate restriction enzyme existing between the initiation site of rep and its natural promoter, p5, the left end of the rep sequence (N-rep) was first amplified by PCR. The sequence of the upper primer was SEQ ID NO:2: TATTTAAGCCCGAGTGAGCT (from position of 255 to 274) which introduced a nucleotide substitution A->T at position 274 (underlined). A SacI site was then generated to permit the cloning of N-rep into pTM1 and in-frame expression of Rep protein from the EMCV UTR preferred initiation site (within the NcoI site). The PCR product (739 bp in length) was directly cloned into pCR2.1 vector (Invitrogen) and named pCR-N-rep.

The pTM-1 plasmid was digested with SacI and Stu I restriction enzymes and ligated with a 3.7 kb SacI/SnaBI fragment from psub201 [Samulski et al, J. Virol., 61:3096-3101 (1987)] containing the right end of the AAV genom (without ITR sequ nce), i.e., the c-

terminal portion of rep and full-length cap sequence. This T7 promoter-driven rep/cap construct is named pT7-c-rep/cap.

The first 535 bp sequence of rep was removed from the pCR-N-Rep plasmid by SacI digestion and subcloned into pT7-C-rep/cap, which has similarly been digested with SacI and subjected to alkaline phosphatase treatment to prevent self-ligation of the vector. The final construct was named pT7 rep/cap which contains the full length coding sequence of rep/cap downstream of T7 promoter/EMCV UTR, followed by the T7 terminating sequence.

B. *Production T7 rep/cap Adenovirus*

pAd.link is a construct similar to pAd.CMV.link, a plasmid containing the adenoviral sequences 0 to 16 map units deleted of E1a and E1b as described in the other adenovirus vectors into which a CMV promoter-polylinker cassette was cloned and described in X. Ye et al, J. Biol. Chem., 271:3639-3646 (1996). However, pAd.link contains no CMV promoter or polyA tail sequence.

The entire region including the T7 promoter, EMCV UTR, rep/cap and T7 terminating sequence was excised from pT7 rep/cap by digestion with ClaI and EagI, and then subcloned into the adenoviral sequences of pAd.link, which had previously been subjected to ClaI/SalI digestion, after filling in the sticky ends of EagI and SalI by Klenow polymerase. The resulting plasmid is designated pAd.T7 rep/cap.

The pAd.T7 rep/cap is co-transfected with the ClaI linearized Ad.del327 backbone DNA into 293 cell for the generation of T7 rep/cap adenovirus.

Example 4 - Cell Line Expressing rep/cap

A cell line stably transfected with pT7 rep/cap is established by transfection of pMTCB6+ into 293 cell at ratio of 10:1 and selected with Geneticin. Each clone is analyzed for the presence of rep protein by transfection with T7 Pol expressing plasmid.

Example 5 - Production of Recombinant AAV Hybrid Vector

Plasmid pAV.CMVLacZ serves as a template for rAAV to be replicated and packaged in the presence of AAV non-structural and capsid proteins.

Plasmid AV.CMV*LacZ* is a rAAV cassette in which rep and cap genes are replaced with a minigene expressing ß-galactosidase from a CMV promoter. The linear arrangement of AV.CMV*LacZ* includes:

(a) the 5' AAV ITR (bp 1-173) obtained by PCR using pAV2 [C. A. Laughlin et al, Gene, 23: 65-73 (1983)] as template [nucleotide numbers 365-538 of SEQ ID NO:1];

(b) a CMV immediate early enhancer/promoter [Boshart et al, Cell, 41:521-530 (1985); nucleotide numbers 563-1157 of SEQ ID NO:1],

(c) an SV40 intron (nucleotide numbers 1178-1179 of SEQ ID NO:1),

(d) *E. coli* beta-galactosidase cDNA (nucleotide numbers 1356 - 4827 of SEQ ID NO:1),

(e) an SV40 polyadenylation signal (a 237 BamHI-BclI restriction fragment containing the cleavage/poly-A signals from both the early and late transcription units; nucleotide numbers 4839 - 5037 of SEQ ID NO:1) and

(f) 3'AAV ITR, obtained from pAV2 as a SnaBI-BglII fragment (nucleotide numbers 5053 - 5221 of SEQ ID NO:1).

Where desired, the LacZ gene can be replaced with a desired therapeutic or other transgene for the purpose of generating new rAAV. See, Fig. 4. The sequence including CMV directed LacZ reporter cassette in between two AAV ITR sequences is excised from pAV.CMV.LacZ by PvuII digestion. This fragment is ligated with the EcoRV treated pAd.link to generate the plasmid pAd.AV.CMVLacZ. This plasmid is co-transfected with ClaI linearized Addel327 backbone DNA to generate an adeno-rAAV hybrid virus.

Example 6 - Cell line containing rescuable, integrated rAAV template

293 cells are transfected/infected with pAV.CMVLacZ/rAAV Ad hybrid virus to generate cell line that has incorporated rAAV, as determined by analysis of the genomic DNA by Southern blot. The clone is examined for the rescue of rAAV template by transfection/infection with rep/cap expressing constructs.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Trustees of the University of Pennsylvania
Wilson, James M.
Chen, Nancie N.

(ii) TITLE OF INVENTION: An Inducible Method for Production of Recombinant Adeno-Associated Viruses Utilizing T7 Polymerase

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
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(F) ZIP: 19477

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: WO
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/024,699
(B) FILING DATE: 06-SEP-1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kodroff, Cathy A.
(B) REGISTRATION NUMBER: 33,980
(C) REFERENCE/DOCKET NUMBER: GNVPN.022CIP1PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 215-540-9200
(B) TELEFAX: 215-540-5818

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10398 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TATTTAAGCC CGAGTGAGCT 20

What is claimed is:

1. A method for production of recombinant adeno-associated virus (AAV) comprising the steps of:

(a) introducing into a selected host cell

a first vector comprising T7 polymerase under control of sequences which drive expression thereof,

a second vector comprising AAV *rep* and *cap* genes under control of T7 promoter sequences which drive expression of rep and cap; and

a third vector comprising from 5' to 3', a cassette consisting essentially of a 5' AAV inverted terminal repeat (ITR), a selected minigene, and a 3' AAV ITR;

(b) culturing the host cell under conditions which permit replication and packaging of recombinant AAV; and

(c) recovering the recombinant AAV.

2. The method according to claim 1 wherein at least one of the vectors is an adenovirus and the host cell is a 293 cell.

3. The method according to claim 1 wherein the first vector is a recombinant adenovirus.

4. The method according to claim 1 wherein the second vector is a recombinant adenovirus.

5. The method according to claim 1 wherein the third vector further comprises adenoviral sequences flanking the cassette.

6. The method according to any of claims 1 to 5 wherein the minigene contains a transgene which is a marker gene.

7. The method according to claim 6 wherein the minigene contains a transgene which is a therapeutic gene.

8. A method for production of recombinant adeno-associated virus (AAV) comprising the steps of:

(a) providing a host cell which expresses T7 polymerase;

(b) introducing into the host cell a first vector which comprises AAV *rep* and *cap* genes under control of T7 promoter sequences;

(c) introducing into the host cell a second vector comprising a cassette consisting essentially of 5' AAV inverse terminal repeat (ITR), a selected minigene, and 3' AAV ITR; and

(d) culturing the host cell under conditions which permit replication and packaging of a recombinant AAV.

9. The method according to claim 8 wherein step (b) comprises the step of transfecting the host cell with a vector comprising the T7 promoter and the AAV *rep* and *cap* genes.

10. The method according to claim 8 wherein step (b) comprises the step of infecting the host cell with a recombinant adenovirus comprising the T7 promoter sequences, and the AAV *rep* and *cap* genes.

11. The method according to claim 8 wherein step (c) comprises transfecting the host cell with a vector comprising the cassette.

12. The method according to claim 8 wherein step (c) comprises infecting the host cell with a recombinant adenovirus comprising the cassette flanked by adenovirus sequences.

13. A method for production of recombinant adeno-associated virus (AAV) comprising the steps of:

(a) providing a host cell stably transfected with AAV *rep* and *cap* genes under control of T7 promoter sequences;

(b) introducing into the host cell a vector comprising T7 polymerase;

(c) introducing into the host cell with vector comprising a cassette consisting essentially of a 5' AAV inverse terminal repeat (ITR), a selected minigene, and a 3' AAV ITR; and

(d) culturing the host cell under conditions which permit replication and packaging of a recombinant AAV.

14. The method according to claim 13 wherein step (b) comprises the step of transfecting the host cell with a vector comprising the T7 polymerase gene.

15. The method according to claim 13 wherein step (b) comprises the step of infecting the host cell with a recombinant adenovirus comprising the T7 polymerase gene under control of regulatory sequences controlling expression thereof.

16. The method according to claim 13 wherein step (c) comprises the step of transfecting the host cell with a vector comprising the cassette.

17. The method according to claim 13 wherein step (c) comprises the step of infecting the host cell with a recombinant adenovirus comprising the cassette flanked by adenovirus sequences.

18. A method for production of recombinant adeno-associated virus (AAV) comprising the steps of:

(a) providing a host cell comprising a cassette consisting essentially of 5' AAV inverse terminal repeats (ITR), a selected minigene, and a 3' AAV ITR;

(b) introducing into the host cell a vector comprising AAV *rep* and *cap* genes under control of T7 promoter sequences;

(c) introducing into the host cell a vector comprising the T7 polymerase; and

(d) culturing the host cell under conditions which permit replication and packaging of a recombinant AAV.

19. The method according to claim 18 wherein step (b) comprises the step of transfecting the host cell with a plasmid vector.

20. The method according to claim 18 wherein step (b) comprises the step of infecting the host cell with a recombinant adenoviral vector.

21. The method according to claim 18 wherein step (c) comprises the step of transfecting the host cell with a plasmid vector containing the T7 polymerase under control of regulatory sequences which direct expression thereof.

22. The method according to claim 18 wherein step (c) comprises the step of infecting the host cell with a recombinant adenovirus comprising the T7 polymerase under control of regulatory sequences which direct expression thereof.

23. A method for production of recombinant adeno-associated virus (AAV) comprising the steps of:

(a) providing a host cell stably transfected with a cassette consisting essentially of 5' AAV inverse terminal repeats (ITR), a selected minigene, and a 3' AAV ITR and a plasmid comprising AAV *rep* and *cap* genes under control of T7 promoter sequences;

(b) introducing into the host cell a vector comprising the T7 polymerase; and

(c) culturing the host cell under conditions which permit replication and packaging of a recombinant AAV.

24. A method for production of recombinant adeno-associated virus (AAV) comprising the steps of;

(a) providing a host cell stably transfected with a cassette consisting essentially of 5' AAV inverse terminal repeats (ITR), a selected minigene, and a 3' AAV ITR and a plasmid comprising T7 polymerase;

(b) introducing into the host cell a vector comprising AAV *rep* and *cap* genes under control of T7 promoter sequences; and

(c) culturing the host cell under conditions which permit replication and packaging of a recombinant AAV.

25. A method for production of recombinant adeno-associated virus (AAV) comprising the steps of:

(a) providing a host cell stably transfected with

(i) a cassette consisting essentially of 5' AAV inverse terminal repeats (ITR), a selected minigene, and a 3' AAV ITR;

(ii) a plasmid comprising T7 polymerase under control of sequences which regulate expression thereof, said sequences comprising an inducible promoter; and

(iii) a plasmid AAV *rep* and *cap* genes under control of T7 promoter sequences; and

(b) inducing expression of said T7 promoter.

26. A recombinant adenovirus produced according to the method of any one of claims 1 - 25.

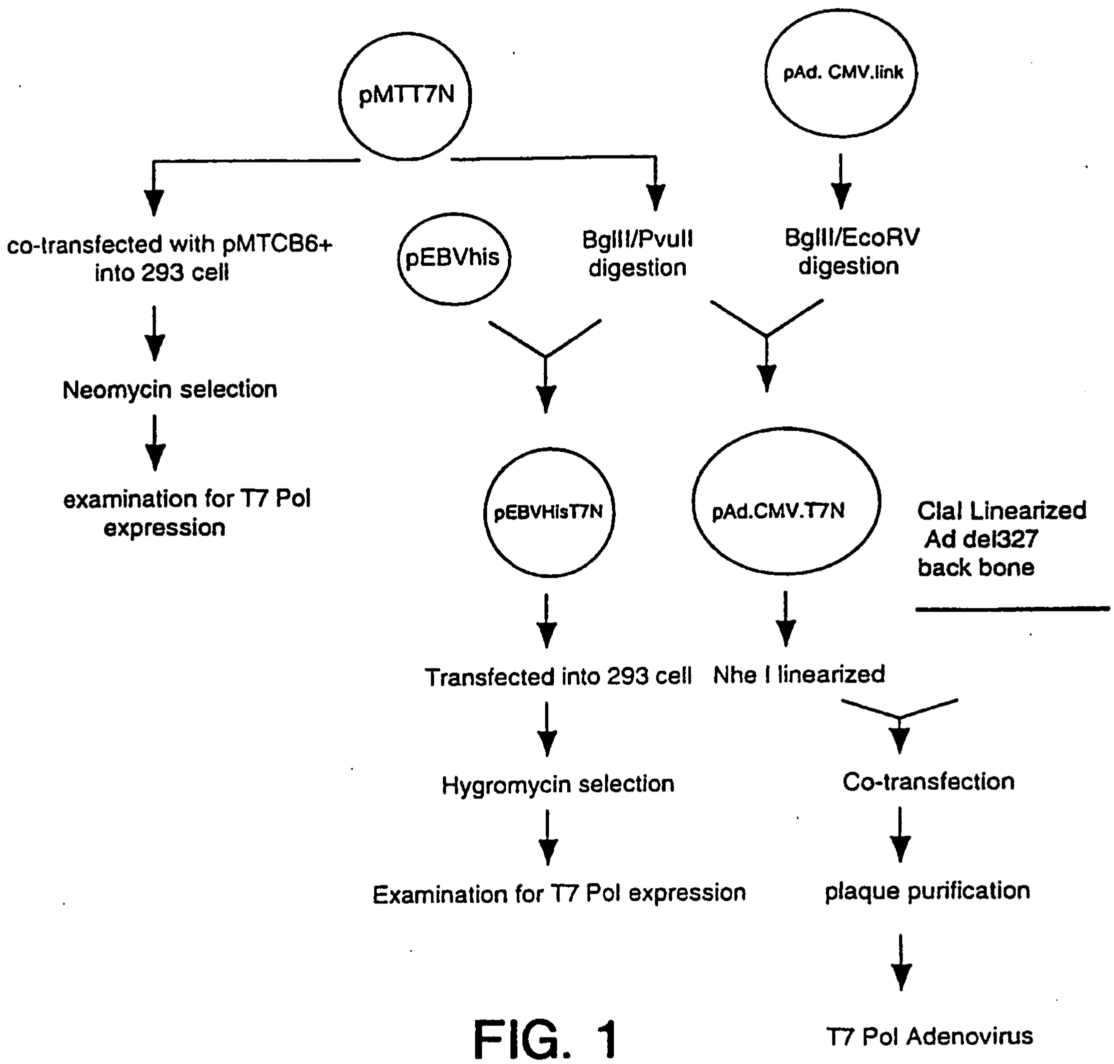
pMTT7N
pAd. CMV.link
co-transfected with pMTCB6+
into 293 cell
pEBVhis
BglII/PvuII
digestion
BglII/EcoRV
digestion
Neomycin selection
examination for T7 Pol
expression
pEBVHisT7N
pAd.CMV.T7N
ClaI Linearized
Ad del327
back bone
Transfected into 293 cell
Nhe I linearized
Hygromycin selection
Co-transfection
Examination for T7 Pol expression
plaque purification
T7 Pol Adenovirus

FIG. 1

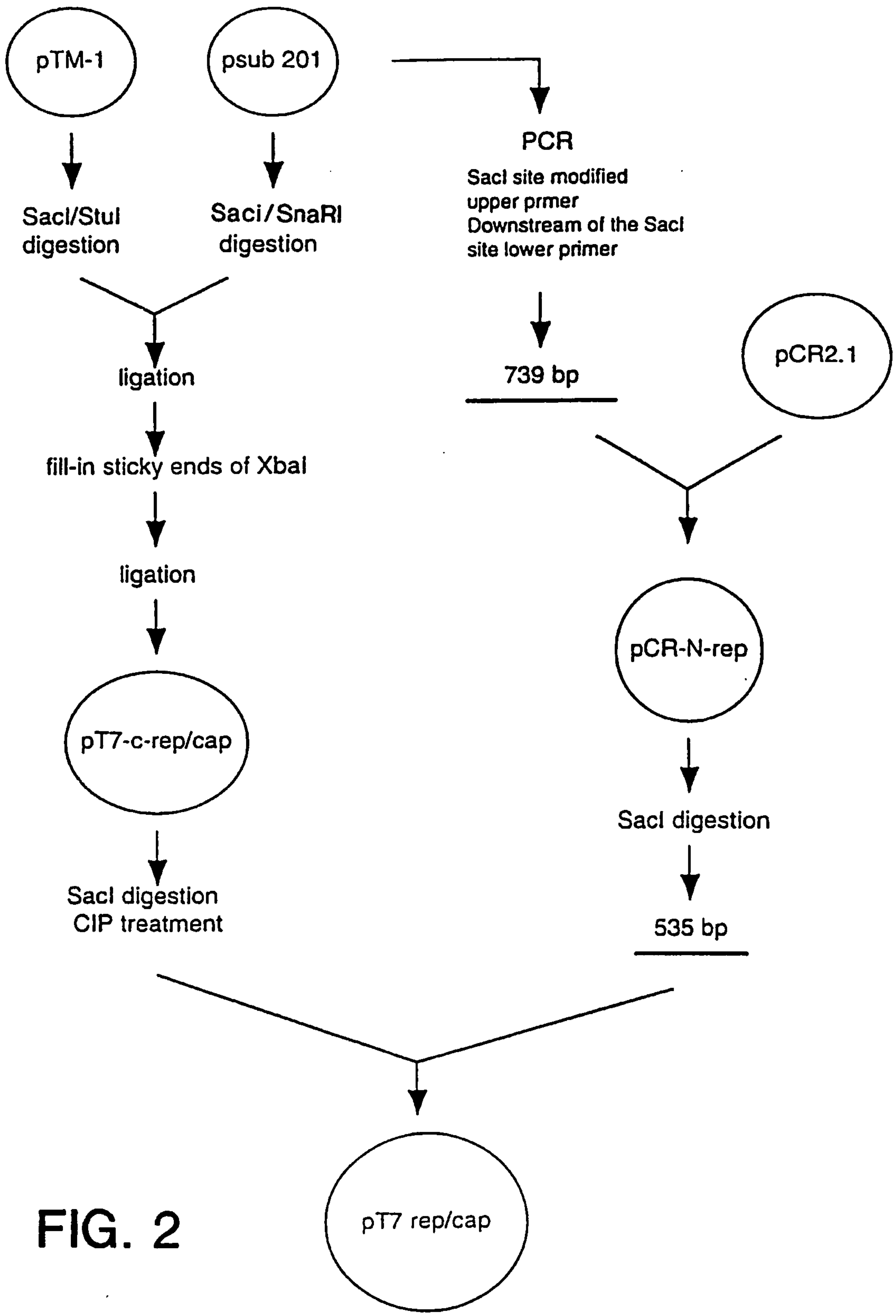
pTM-1
psub 201
SacI/StuI digestion
Saci/SnaRI digestion
PCR
SacI site modified upper prmer
Downstream of the SacI site lower primer
ligation
fill-in sticky ends of XbaI
ligation
739 bp
pCR2.1
pCR-N-rep
pT7-c-rep/cap
SacI digestion
535 bp
SacI digestion
CIP treatment
pT7 rep/cap

FIG. 2

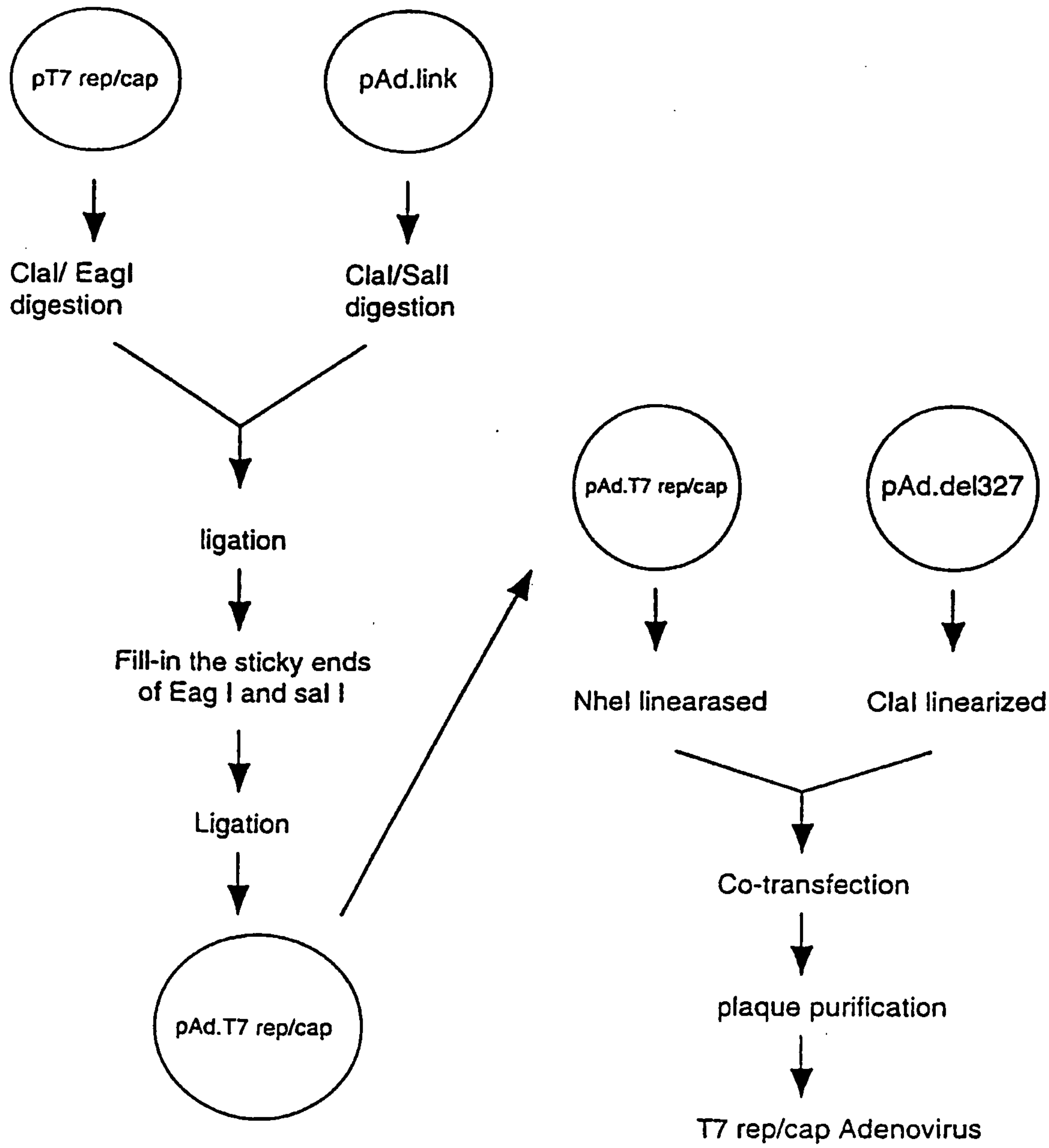
pT7 rep/cap
pAd.link
ClaI/ EagI digestion
ClaI/SalI digestion
ligation
Fill-in the sticky ends of Eag I and sal I
Ligation
pAd.T7 rep/cap
pAd.T7 rep/cap
pAd.del327
NheI linearased
ClaI linearized
Co-transfection
plaque purification
T7 rep/cap Adenovirus

FIG. 3

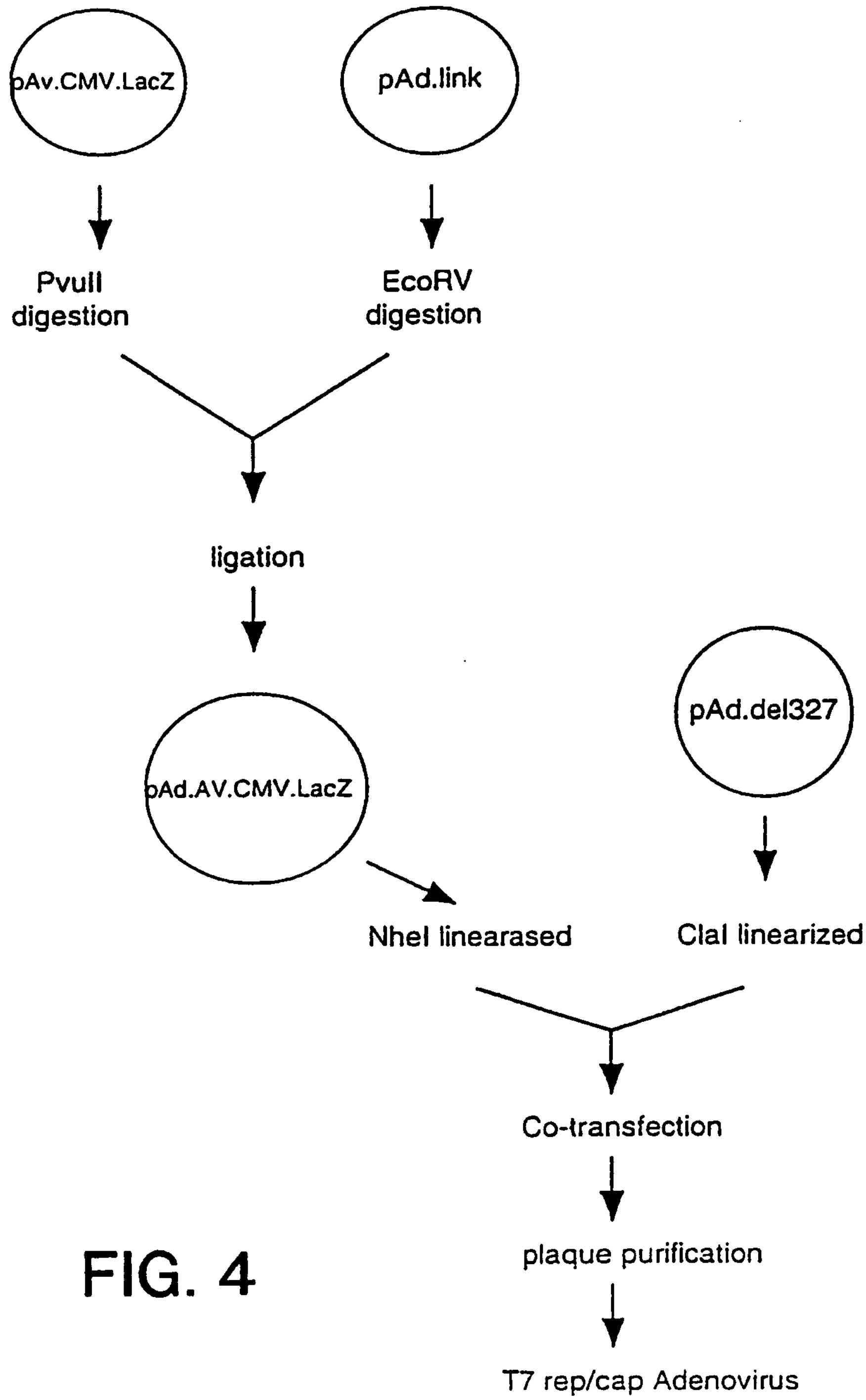
pAv.CMV.LacZ
pAd.link
PvuII digestion
EcoRV digestion
ligation
pAd.AV.CMV.LacZ
pAd.del327
NheI linearased
ClaI linearized
Co-transfection
plaque purification
T7 rep/cap Adenovirus

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/15716

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N7/01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEONARD, C. J. ET AL: "Cloning, expression, and partial purification of Rep78: an adeno - associated virus replication protein" VIROLOGY (1994), 200(2), 566-73 CODEN: VIRLAX;ISSN: 0042-6822, 1994, XP002052542 see the whole document ---	26
X	WO 95 13392 A (OHIO MED COLLEGE ;TARGETED GENETICS CORP (US); TREMPE JAMES P (US)) 18 May 1995 see page 8, line 16 - page 9, line 1; claims 1-18 ---	26
X	WO 96 17947 A (TARGETED GENETICS CORP ;ALLEN JAMES M (US)) 13 June 1996 see the whole document ---	26

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search	Date of mailing of the international search report
19 January 1998	30/01/1998

Name and mailing address of the ISA	Authorized officer
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X	WO 95 14771 A (US HEALTH ;GENETIC THERAPY INC (US)) 1 June 1995 see the whole document ---	26
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X	WO 94 13788 A (UNIV PITTSBURGH) 23 June 1994 see the whole document ---	26
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A	WO 94 26911 A (UNIV OHIO) 24 November 1994 see the whole document ---	1-26
A	WO 91 00905 A (US ARMY) 24 January 1991 see the whole document --- -/--	1-26

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Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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International Application No
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